



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : A01N 1/02, C12N 5/06	A1	(11) International Publication Number: WO 91/07086 (43) International Publication Date: 30 May 1991 (30.05.91)
(21) International Application Number: PCT/US90/06347 (22) International Filing Date: 7 November 1990 (07.11.90) (30) Priority data: 433,952 9 November 1989 (09.11.89) US (71) Applicant: CRYOLIFE, INC. [US/US]; 2211 New Market Parkway, Marietta, GA 30067 (US). (72) Inventors: BROCKBANK, Kelvin, G., M. ; 3544 West Hampton Drive, Marietta, GA 30064 (US). ANDEREGG, Katherine, A. ; 5300 5th Avenue, Pittsburgh, PA 15232 (US). (74) Agents: KOKULIS, Paul, N. et al.; Cushman, Darby & Cushman, Eleventh Floor, 1615 L. Street, N.W., Washington, DC 20036 (US).		(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent), SU. Published <i>With international search report.</i>
(54) Title: STORAGE SOLUTION (57) Abstract The present invention relates to a method of maintaining viability of a cell, tissue or organ. The method involves maintaining the cell, tissue or organ in contact with a solution comprising transferrin and selenium at a sub-ambient temperature in a non-frozen state. The invention further relates to a solution suitable for use in the above-described method.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
BJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LJ	Liechtenstein	SU	Soviet Union
CI	Côte d'Ivoire	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

STORAGE SOLUTION

BACKGROUND OF THE INVENTIONTechnical Field

5 The present invention relates in general,
to the preservation of organs, tissues and cells
during storage and transport and, in particular, to
a method of maintaining organs, tissues and cells in
a viable state prior to transplantation, and to a
composition suitable for use in such a method.

10 Background Information

Ischemia, a localized tissue hypoxia
resulting from partial or complete loss of blood
circulation, ensues rapidly upon death of an
organism. In designing a protocol for storing a
15 tissue prior to transplantation, the susceptibility
of the particular tissue to ischemia must be
considered. One factor that influences the rate at
which ischemia produces cellular injury, and
subsequently cell death, is temperature. Kidneys,
20 for example must be procured immediately after
cessation of donor heartbeat, and can be stored for
only 1-3 days at 0°-4°C, using current technology.
The exact time is dependant upon whether or not
continuous perfusion is employed. This is in
25 contrast with bone marrow, which can tolerate at
least 12 hours of warm ischemia post mortem and 3
days of cold ischemia at 0°-4°C.

In order to extend the period for which
particular cells, tissues and organs can be
30 maintained in a state which will permit subsequent

successful transplantation into a recipient host, new methods must be developed. One such method is provided by the present invention.

SUMMARY OF THE INVENTION

5 It is a general object of the invention to provide a method of maintaining cells, tissues and organs in a viable state.

 It is a specific object of the invention to provide a method of storing cells, tissues and
10 organs at refrigerated temperatures prior to transplantation.

 It is another object of the present invention to provide a tissue storage solution that permits storage of cells, tissues and organs at
15 refrigerated temperatures for periods of time longer than is possible using present clinically accepted solutions.

 Further objects and advantages of the present invention will be clear from a reading of
20 the description that follows.

 The present invention relates to a method of delaying the detrimental effects of ischemia on organ, tissue and cell viability, and to a storage solution suitable for use in such a method.

25 In one embodiment, the present invention relates to a method of storage comprising the steps of:

 i) contacting a cell, tissue or organ to be stored with a solution comprising transferrin and
30 selenium; and

ii) maintaining the cell, tissue or organ in contact with the solution at a sub-ambient temperature in a non-frozen state.

In another embodiment, the present invention relates to a storage solution comprising insulin, transferrin, hydrocortisone, selenium and a Goodes buffer, for example, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES).

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1: Comparison of six cold storage solutions using canine anterior cruciate ligament-derived fibroblasts in vitro.

FIGURE 2: Cold ischemia study using hydrocortisone, insulin, transferrin and selenium-containing storage solution and Euro-Collins storage solution.

FIGURE 3: Effects of hydrocortisone, insulin, transferrin and selenium removal on cell survival at 4°C.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a method of storing cells, tissues and organs, and to a storage solution suitable for use in such a method. The present storage method is such that viability of the stored material is maintained. Maintenance of viability permits subsequent successful

transplantation of the stored material into a recipient host.

In the present method, material to be stored is placed in contact with a storage solution comprising transferrin and selenium, advantageously at concentrations in the ranges of 2.5 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$ and 2.5 ng/ml to 7.5 ng/ml , respectively. In a preferred embodiment, insulin and hydrocortisone are also present in the storage solution, advantageously at concentrations in the ranges of 2.5 $\mu\text{g/ml}$ to 7.5 $\mu\text{g/ml}$ and 25 ng/ml to 40 ng/ml , respectively. In a most preferred embodiment, a Goodes buffer, for example HEPES, is also present in the solution at a concentration in the range of 10mM to 30 mM (corresponding to 2.92-8.85 g/l in the case of HEPES). The inclusion of a Goodes buffer in the storage solution is particularly advantageous where storage for more than two days is required. For shorter storage periods, other pharmaceutically acceptable buffers, for example, a bicarbonate buffer, can be used.

The above-described components of the tissue storage solution of the present invention can be present, for example, in a medium capable of supporting cellular metabolism in vitro at 37°C or the components can be present in a buffered physiological salt solution that is incapable of supporting cellular metabolism at 37°C. Such salt solutions can include a carbohydrate source (for example, glucose).

In the present method, cell viability is maintained by storing the cells, tissues or organs in the above-described solution at sub-ambient

temperatures, in a non-frozen state.

Advantageously, temperatures in the range of -4° to 4°C are used.

Materials suitable for storage according to the present method include, but are not limited to, heart, kidney, lung, liver, cornea, pancreas, skin, blood vessels, tendons, ligaments, bone, bone marrow, endocrine and exocrine glands, gametes, ova, nerves, gastrointestinal tract, ureter, bladder, or structures or cellular components derived from any of the above. Where intact organs are to be stored, such organs are flushed with the above-described solution prior to storage.

The following non-limiting Examples further describe the present invention.

EXAMPLE 1

Comparison of Six Storage Solutions

Confluent cultures of canine anterior cruciate ligament(ACL)-derived fibroblasts were placed, for five days at 0° - 4°C , in: 1) physiological saline (0.9% (w/v) NaCl) (designated NaCl); 2) a culture medium containing fetal calf serum (Dulbecco's Modified Eagle Medium plus 10% v/v fetal calf serum) (designated PS); 3) a buffered physiological salt solution (0.10 g/l CaCl_2 (anhydrous), 0.20 g/l KCl, 0.20 g/l KH_2PO_4 , 0.10 g/l $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 8.00 g/l NaCl, and 2.16 g/l $\text{Na}_2\text{HPO}_4 \cdot \text{H}_2\text{O}$) (designated DPBS); 4) Hanks Balanced Salt Solution as in Table I without hydrocortisone, insulin, transferrin and selenium and containing 15mM HEPES

instead of 25mM (designated H); 5) the solution of Table I (designated HITS); or 6) the solution of Table I plus chondroitin sulfate (25 g/l) and sucrose (47.922 g/l) (designated HITS').

5 At the end of this period, the solutions were removed from the cultures, the cells were washed, and placed in contact with fresh serum-free culture medium. After 2 hrs. of incubation in the serum-free medium, the cells were labeled with
10 tritiated glycine at 37°C in a 5% CO₂ and air incubator using a technique adopted from that detailed below in Example 2. The relative protein incorporation between the experimental groups is an indication of the level of cellular viability.

15

Table I

	<u>g/l</u>
CaCl ₂ (anhydrous)	0.14
KCl	0.40
KH ₂ PO ₄	0.06
20 MgCl ₂ ·6H ₂ O	0.10
MgSO ₄ ·7H ₂ O	0.10
NaCl	8.00
NaHCO ₃	0.35
Na ₂ HPO ₄ ·7H ₂ O	0.09
25 D-Glucose	1.00
Phenol Red	0.01
Hepes	4.425
Transferrin	0.005
Insulin	0.005
30 Selenium	0.000005
Hydrocortisone	0.000036

The results shown in Figure 1 clearly indicate the superiority of the solution of the present invention (that given in Table I) over the others tested. (C = control - no storage at 0°-
5 4°C; storage medium = Dulbecco's Modified Eagle Medium plus 10% v/v fetal calf serum.

EXAMPLE 2

Comparison of Hydrocortisone, 10 Insulin, Transferrin and Selenium- Containing Storage Solution and Euro- Collins Storage Solution

Bisected human heart valve leaflets were placed in either the solution described in Table I or Euro-Collins solution at 4°C for 1-5 days.
15 (Euro-Collins = KH_2PO_4 (2.05 g/l), KHP0_4 (7.40 g/l), KCl (1.12 g/l), NaCO_3 (0.84 g/l), and glucose (38.5 g/l).) After cold storage, the leaflets were washed and placed in tritiated glycine and incorporation of the isotope into protein was determine after 48 hrs
20 of incubation using the following protocol:

Tritiated Labelling of Tissue

Day 1 - Make up a $16\mu\text{Ci/ml}$ ^3H -glycine solution in serum free Dulbecco's Modified Eagle Medium (DMEM). Cut tissue up into small pieces and
25 place in a 5 ml snap-cap tube. Add 0.5 ml of the ^3H -glycine to each tube. Incubate for 48 hours at 37°C.

Day 2 - Decant medium from the tubes and wash tissue quickly twice with phosphate buffered
30 saline (PBS). Add PBS and let sit for 30 minutes.

Remove PBS and add more PBS. Incubate overnight at 4°C.

Day 3 - Decant PBS off of tissue and place tissue into 15 x 100mm glass tubes. Wash for 15 minutes in alcohol, then wash for 15 minutes with ether. Remove ether and allow the tissue to dry for at least one hour. Weigh and record weight of tissue from each tube. Place all tissue into clean 12 x 75 mm glass tubes.

Add 200 μ l H₂O to each tube and let rehydrate for 30 minutes to 1 hour.

Add 500 μ l 1M NaOH to each tube and place tubes in a heating block at 60°C. Allow 60 minutes incubation for valve tissue.

Pipette samples into microtubes and sonicate twice for 20 seconds each time. Centrifuge in microfuge for 2 minutes.

Apply 100 μ l of each sample to glass fiber filter discs. Allow to dry for at least one hour. Move filter discs to glass scintillation vials and add 2ml ice cold 10% trichloroacetic acid (TCA) to each vial. Refrigerate for 30 minutes minimum. Remove TCA and wash four times with 3 ml ice cold alcohol. Then wash twice with 3 ml ice cold ether.

Allow filter discs to dry for at least one hour. Add 130 μ l H₂O to each filter disc. Add 1 ml Protosol to each vial. Vortex vigorously. Add 10 ml scintillation fluid and 100 μ l glacial acetic acid. Transfer vials into racks, place racks in counter and allow to dark adapt for 30 minutes before counting. Count each vial for 5 minutes.

The results are summarized in Figure 2. A total of 27 comparisons were done after 1-5 days of

incubation. In 23 out of the 27 comparisons, the solution described in Table I was clearly superior. In only one instance did the Euro-Collins solution support protein synthesis levels greater than the solution described in Table I. In three cases, the numbers were similar.

These results demonstrate a highly significant maintenance of cellular viability by the storage solution of the present invention, relative to the current clinically accepted alternative.

EXAMPLE 3

Effects of Removal of Hydrocortisone, Insulin, Transferrin and Selenium on Cell Storage at 4°C

Human kidney-derived proximal tubule cells were plated on bovine type I collagen/fetal calf serum-coated Costar 24 well plates and placed in either complete solution (C) (see Table II) or in the solution shown in Table I from which either one or all of the following had been removed: hydrocortisone (H), insulin (I), transferrin (T), or selenium (S). The plates were then placed in a refrigerator at 4°C for 72 hours. Viability was assayed by the neutral red spectrophotometric assay described below in Example 4.

Table II

Complex culture medium used for testing HITS components.

COMPONENTS

	mg/L		
INORGANIC SALTS:			
CaCl ₂ (anhyd.)	100		
CaCl ₂ ·2H ₂ O	22		
CuSO ₄ ·5H ₂ O	0.001245		
FeSO ₄ ·7H ₂ O	0.417		
KCl	311.8		
MgCl ₂ ·6H ₂ O	61		
MgSO ₄ ·7H ₂ O	100		
NaCl	6999.5		
NaHCO ₃	2438		
Na ₂ HPO ₄ ·7H ₂ O	134		
ZnSO ₄ ·7H ₂ O	0.4315		
Fe(NO ₃) ₃ ·9H ₂ O	0.05		
NaH ₂ PO ₄ ·H ₂ O	62.5		
		Vitamins	
		Biotin	0.00365
		D-Capantothenate	2.24
		Choline chloride	8.98
		Folic acid	2.65
		i-Inositol	12.60
		Niacinamide	2.0185
		Pyridoxine HCl	0.031
		Riboflavin	0.219
		Thiamine HCl	2.17
		Vitamin B ₁₂	0.68
		Pyridoxal HCl	2.0

OTHER COMPONENTS

D-Glucose	1401
Hypoxanthine	2.05
Linoleic acid	0.042
Lipoic acid	0.105
Phenol red	8.1
Pantothine 2HCl	0.0805
Sodium pyruvate	110
Thymidine	0.365

TABLE II (continued)

AMINO ACIDS:

L-Alanine	4.45
L-Arginine HCl	147.5
L-Asparagine.H ₂ O	7.505
L-Aspartic acid	6.65
L-Cysteine	24
L-Cysteine HCl.H ₂ O	17.56
L-Glutamic acid	7.35
L-Glutamine	365
Glycine	18.75
L-Histidine HCl.H ₂ O	31.48
L-Isoleucine	54.47
L-Leucine	59.05
L-Lysine HCl	91.25
L-Methionine	17.24
L-Phenylalanine	35.48
L-Proline	17.25
L-Serine	26.25
L-Threonine	53.45
L-Tryptophan	9.02
L-Tyrosine	38.70
L-Tyrosine (disodium salt)	-
L-Valine	52.85

Data shown in Figure 3 are expressed as the mean survival \pm 1 S.E. of 4-6 experiments in percent of 37°C controls.

EXAMPLE 4

5 Influence of Divalent Cations (Ca^{2+} and Mg^{2+}) on Cell Viability in the Presence of HEPES and Bicarbonate Buffers.

10 In order to access the effects of HEPES and bicarbonate buffers on the viability of cells stored in the absence of divalent cations, human proximal tubule cells were plated on bovine type 1 collagen/fetal calf serum coated Costar 24 well plates and placed in the solutions indicated below in Table III (which solutions are based on that
15 shown in Table I) for 24, 48, or 72 hours under cold (4°C) ischemic conditions. N=4 for each solution in each of the four experiments performed. All data is expressed in Table III as a percent survival compared to a 37°C control as assessed by the
20 neutral red spectrophotometric assay described as follows:

Neutral Red Assay: Highest quality Neutral Red dye was obtained from Aldrich Chemical Co. (Milwaukee, WI). A 0.5% solution was prepared in tris-buffered
25 saline (TBS). A ten fold concentrated TBS stock was prepared as follows: to 1.0 liter of distilled deionized water was added 24.2 g Trizma 7.7 (Sigma Chemical Co., St. Louis, MO), 68.0 g NaCl, 2.0 g KCl, 2.0 g $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 1.0 g CaCl_2 (anhydrous).
30 The saline was then filter sterilized using a 0.22 μ

nitrocellulose filter. The saline stock was diluted to 1X using distilled deionized water. To prepare the Neutral Red solution, 0.5g Neutral Red was added to 100 ml 1X TBS solution, care being taken to minimize the light exposure of this photosensitive dye. The dye solution was filtered using Whatman No. 42 paper just prior to use.

Kidney tubule cells were gently washed 4X with TBS which had been warmed to 37°C. Following suction removal of the last saline wash, 0.5 ml of 0.5% Neutral Red in TBS warmed to 37°C was added to each well. The plates were then floated in a covered 37°C water bath for 30 min to allow maximum dye uptake. The unabsorbed Neutral Red solution was then aspirated off and the cells were washed 4X with cold TBS at 4°C. The dye was extracted with cold 50% ethanol at 4°C for 15 min. A 0.15 ml aliquot was then drawn from each well and placed in the wells of Costar 96-well flat-bottom plates. Controls consisted of 50% ethanol blanks and 1X TBS blanks. Samples were read using a 450 nm filter on a Titertek Multiskan ELISA plate reader. Three rows of serial dye dilutions in 50% ethanol were used to generate a concentration curve.

25

Table III

	BUFFER	CATIONS	DAY 1	DAY 2	DAY 3
	HEPES	Ca, Mg	74%	61%	41%
	HEPES	Ca	98%	93%	80%
	HEPES	Mg	76%	60%	44%
30	HEPES	NONE	100%	97%	88%

14

	HCO ₃ ⁻	Ca, Mg	79%	65%	45%
	HCO ₃ ⁻	Ca	97%	89%	52%
	HCO ₃ ⁻	Mg	80%	64%	48%
5	HCO ₃ ⁻	NONE	99%	92%	59%

Ca²⁺ = CaCl₂ (anhydrous) (0.14 g/l)
 Mg²⁺ = MgCl₂.6H₂O [0.10 g/l] and MgSO₄.7H₂O [0.10 g/l]
 HEPES = 5.66 g/l (20 mM)
 10 HCO₃⁻ = NaHCO₃ [0.35 g/l]

These results clearly demonstrate the superiority of
 HEPES buffer in the absence of magnesium or both
 calcium and magnesium after 3 days of storage.

15

* * * * *

The foregoing invention has been
 described in some detail for purposes of clarity and
 understanding. It will be clear to one skilled in
 the art from a reading of the present disclosure
 that various changes can be made in form and detail
 without departing from the true scope of the
 5 invention.

CLAIMS:

1. A method of maintaining viability of a cell, tissue or organ comprising:

5 i) contacting said cell, tissue or organ with a solution comprising transferrin and selenium; and

ii) maintaining said cell, tissue or organ in contact with said solution at a sub-ambient temperature in a non-frozen state.

10 2. The method according to claim 1 wherein said temperature is about 4°C or less.

3. The method according to claim 1 wherein transferrin is present in said solution at a concentration in the range of 2.5 µg/ml to 10 µg/ml.

15 4. The method according to claim 3 wherein transferrin is present in said solution at a concentration of about 5 µg/ml.

20 5. The method according to claim 1 wherein selenium is present in said solution at a concentration in the range of 2.5 ng/ml to 7.5 ng/ml.

6. The method according to claim 5 wherein selenium is present in said solution at a concentration of about 5.0 ng/ml.

25 7. The method according to claim 1 wherein said solution further comprises insulin.

8. The method according to claim 7 wherein insulin is present in said solution at a concentration in the range of 2.5 $\mu\text{g/ml}$ to 7.5 $\mu\text{g/ml}$.

5 9. The method according to claim 8 wherein insulin is present in said solution at a concentration of about 5 $\mu\text{g/ml}$.

10 10. The method according to claim 1 wherein said solution further comprises hydrocortisone.

11. The method according to claim 10 wherein hydrocortisone is present in said solution at a concentration in the range of 25 ng/ml to 40 ng/ml .

15 12. The method according to claim 11 wherein hydrocortisone is present in said solution at a concentration of about 36 ng/ml .

20 13. A storage solution comprising insulin, transferrin, hydrocortisone, selenium and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

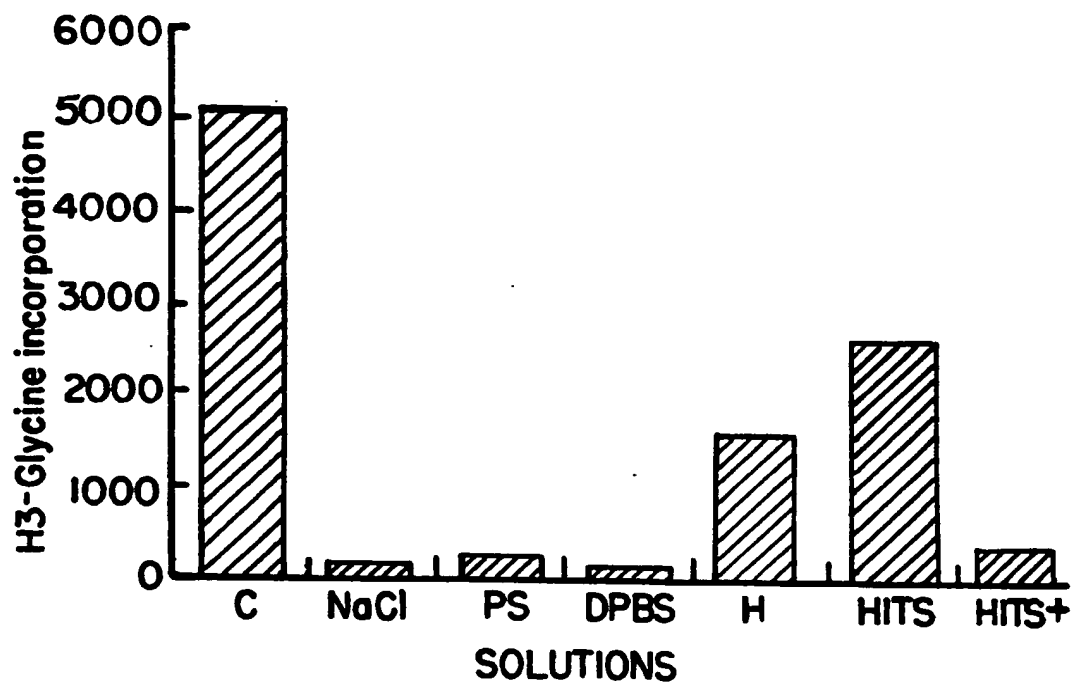
25 14. The storage solution according to claim 13 wherein insulin, transferrin, hydrocortisone, selenium and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid are present in said solution at concentrations in the ranges of 2.5 $\mu\text{g/ml}$ to 7.5 $\mu\text{g/ml}$, 2.5 $\mu\text{g/ml}$ to 10 $\mu\text{g/ml}$, 25 ng/ml

to 40 ng/ml, 2.5 ng/ml to 7.5 ng/ml and 2.95 g/l to 8.85 g/l, respectively.

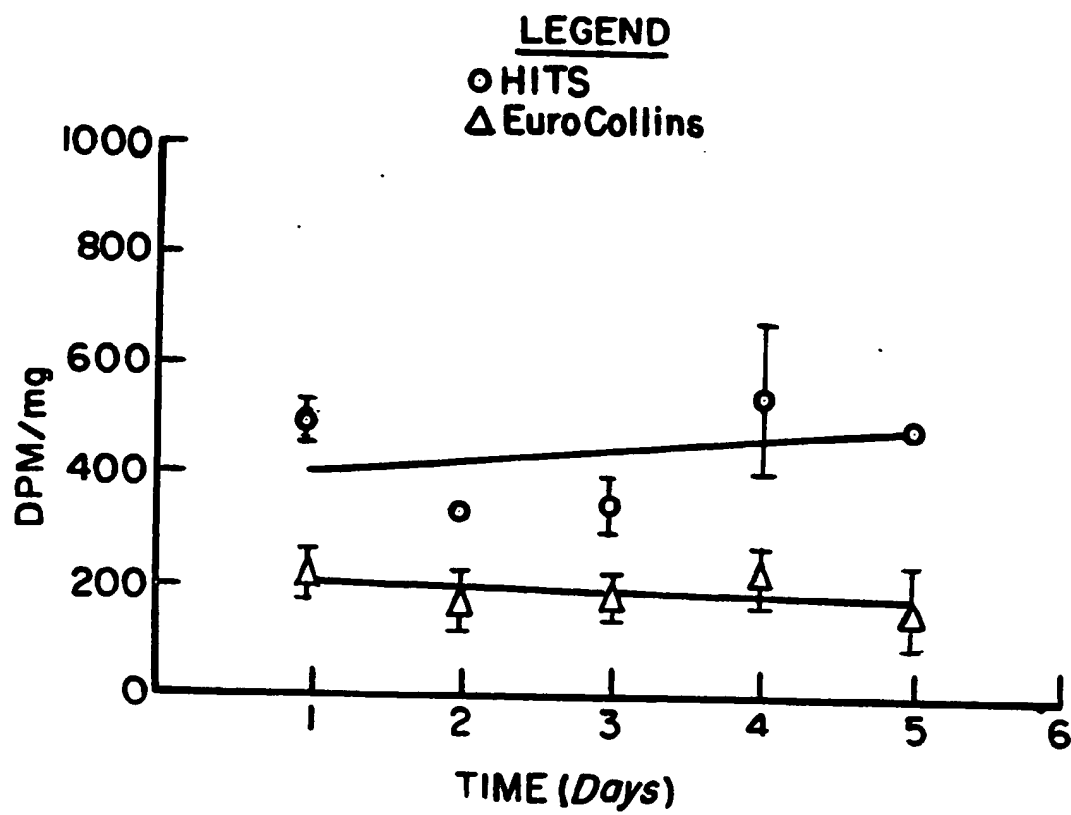
15. The storage solution according to claim 14 wherein insulin, transferrin, hydrocortisone, selenium and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid are present in said solution at concentrations of about 5 μ g/ml, about 5 μ g/ml, about 36 ng/ml, about 5 ng/ml, and about 4.425 g/l, respectively.

16. A storage solution comprising insulin at a concentration in the range of 2.5 μ g/ml to 7.5 μ g/ml, transferrin at a concentration in the range of 2.5 μ g/ml to 10 μ g/ml, hydrocortisone at a concentration in the range of 25 ng/ml to 40 ng/ml, and selenium at a concentration in the range of 2.5 ng/ml to 7.5 ng/ml.

1 / 3

*F I G. I*

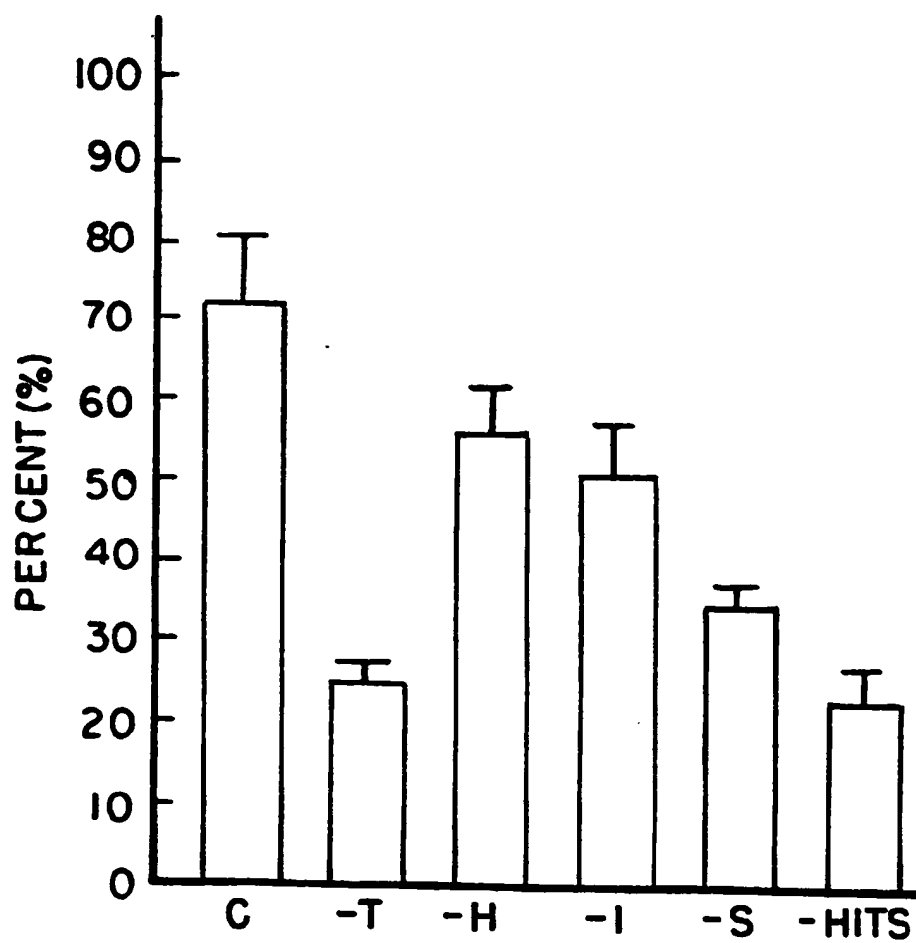
2 / 3



F / G. 2

SUBSTITUTE SHEET

3 / 3

*F I G. 3*

SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No **PCT/US90/06347**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5): A01N 1/02, C12N 5/06 U.S. CL.: 435/1,2,240.2, 240.3, 240.31								
II. FIELDS SEARCHED <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Minimum Documentation Searched ⁴</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; text-align: left; border-bottom: 1px solid black;">Classification System</th> <th style="width: 80%; text-align: left; border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="padding: 5px;">U.S.</td> <td style="padding: 5px;">435/1,2,240.2, 240.3, 240.31</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵</div> <p style="padding: 5px;">Chemical Abstracts Vol. 96-110 (1982-1989) "Transferrin", "Insulin" "Selenium"</p>			Classification System	Classification Symbols	U.S.	435/1,2,240.2, 240.3, 240.31		
Classification System	Classification Symbols							
U.S.	435/1,2,240.2, 240.3, 240.31							
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; text-align: left; border-bottom: 1px solid black;">Category ⁶</th> <th style="width: 60%; text-align: left; border-bottom: 1px solid black;">Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷</th> <th style="width: 30%; text-align: left; border-bottom: 1px solid black;">Relevant to Claim No. ¹⁸</th> </tr> <tr> <td style="vertical-align: top; padding: 5px;"> <u>Y</u> A </td> <td style="vertical-align: top; padding: 5px;"> In Vitro, Volume 19, No. 6, Issued June 1983, Mohamed et al, A Serum-Free Chemically Defined Medium and for Growth of Primary Neonatal Rat Heart Cell Cultures," pages 471-478, see entire document. </td> <td style="vertical-align: top; padding: 5px;"> <div style="text-align: center;"> <u>1-12</u> <u>13-16</u> </div> </td> </tr> </table>			Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸	<u>Y</u> A	In Vitro, Volume 19, No. 6, Issued June 1983, Mohamed et al, A Serum-Free Chemically Defined Medium and for Growth of Primary Neonatal Rat Heart Cell Cultures," pages 471-478, see entire document.	<div style="text-align: center;"> <u>1-12</u> <u>13-16</u> </div>
Category ⁶	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸						
<u>Y</u> A	In Vitro, Volume 19, No. 6, Issued June 1983, Mohamed et al, A Serum-Free Chemically Defined Medium and for Growth of Primary Neonatal Rat Heart Cell Cultures," pages 471-478, see entire document.	<div style="text-align: center;"> <u>1-12</u> <u>13-16</u> </div>						
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"Δ" document member of the same patent family</p> </div> </div>								
IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; padding: 5px;"> Date of the Actual Completion of the International Search ¹⁹ <div style="text-align: center; font-size: 1.2em;">04 January 1991</div> </td> <td style="width: 50%; padding: 5px;"> Date of Mailing of this International Search Report ²⁰ <div style="text-align: center; font-size: 1.5em;">05 FEB 1991</div> </td> </tr> <tr> <td style="padding: 5px;"> International Searching Authority ¹ ISA/US </td> <td style="padding: 5px;"> Signature of Authorized Officer ²⁰ <div style="text-align: center;"> SAM ROSEN </div> </td> </tr> </table>			Date of the Actual Completion of the International Search ¹⁹ <div style="text-align: center; font-size: 1.2em;">04 January 1991</div>	Date of Mailing of this International Search Report ²⁰ <div style="text-align: center; font-size: 1.5em;">05 FEB 1991</div>	International Searching Authority ¹ ISA/US	Signature of Authorized Officer ²⁰ <div style="text-align: center;"> SAM ROSEN </div>		
Date of the Actual Completion of the International Search ¹⁹ <div style="text-align: center; font-size: 1.2em;">04 January 1991</div>	Date of Mailing of this International Search Report ²⁰ <div style="text-align: center; font-size: 1.5em;">05 FEB 1991</div>							
International Searching Authority ¹ ISA/US	Signature of Authorized Officer ²⁰ <div style="text-align: center;"> SAM ROSEN </div>							

ebw 1/91